Halotolerant bacteria in the efflorescences of a deteriorated church

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ABSTRACT: A study on the composition of the efflorescences from the Church of Saint Jerome, Granada, Spain, and their influence on the distribution of the bacterial communities was carried out. The composition of the efflorescences varied depending on the location of the sampling point. The colony forming units (cfu) was related with the type of salt, with a clear difference between halite and epsomite/hexahydrite. The most abundant genera were *Bacillus* and *Micrococcus* and the abundance of bacilli could be explained by their osmotic adaptation to halophilic environments.

RESUMEN: Se ha determinado la composición de las eflorescencias de la iglesia de San Jerónimo, en Granada, España, y relacionado con la presencia de comunidades específicas de bacterias. Existe una conexión entre el tipo de sales y las comunidades bacterianas; así, la epsomita, la sal más abundante en el templo, origina la selección de comunidades capaces de crecer a concentraciones de hasta 15% de esta sal. Sin embargo, las bacterias que basan su halotolerancia en la halita presentan un crecimiento comparativamente menor y no son significativamente estimuladas por la presencia de epsomita. Los géneros más abundantes son *Bacillus* y *Micrococcus*. La abundancia de bacilos se debe a su adaptación osmótica a ambientes salinos.

1 INTRODUCTION

As soon as a rock is extracted from the quarry and used as building material its deterioration starts. Weathering of exposed stone and masonry results from physical, chemical and biological processes, which includes dissolution of carbonates and sulfates, solubilization by leaching of elements from silicates, weathering due to crystallization and hydration pressures, microbial attack by inorganic and organic acids, etc.

A variety of different hygroscopic salts, such as carbonates, chlorides, nitrates, sulfates, etc. can be found in the surfaces of decayed monuments. This means that some monuments with abundant efflorescence deposits can be considered as extremely saline environments. The salinity is not necessarily based on halite, as sometimes epsomite or gypsum are the main components of efflorescences (Cardell & Rodríguez-Gordillo 1992, Incerti et al. 1997).

Halophilic archaea (halobacteria) were known only as inhabiting hostile environments (acid hot springs, hypersaline, or strictly anoxic conditions), but the introduction of molecular techniques in microbial ecology provided a richer census of microbial life.

Norton (1992) stated that halobacteria will be found in any location where the basic requirement for a high concentration of sodium ion is met, and a variety of halophilic archaea have been isolated from salt efflorescences on the walls of salt mining tunnels.

While considerable research has been carried out on halobacteria in salt ponds and other aquatic hypersaline environments (Rodriguez-Valera 1988, Vreeland & Hochstein 1993), very little information exists for terrestrial environments, from which a largely neglected niche with high salt concentrations is found in deteriorated monuments.

In spite of the high number of studies on microbial communities in monuments, halophilic and halotolerant bacteria have not been isolated from culture media, nor detailed search for these groups have been carried out. This seems unusual because as early as in 1935, Hof reported that most of the important groups of bacteria were able to grow in concentrations up to about 15% of salt and that many groups were physiologically active even at much higher salt concentrations. To this omission probably contributed the use of unsuitable media for isolation which rarely included an adequate salt concentration, and/or the non-culturability of bacteria in common media. As a consequence, halophilic and/or halotolerant microbial communities tended to be overlooked.

Recently, stone efflorescences (Incerti et al. 1997) and deteriorated mural paintings (Rölleke et al. 1996) were considered as a potential interesting habitat for archaea. These reports addressed the attention to the search for halophilic communities in deteriorated monuments. In this paper a search for these communities was carried out in the church of Saint Jerome, Granada, Spain.

In a detailed study, Cardell (1998) investigated the chemical composition of the abundant efflorescences in this church, and therefore it is possible to correlate bacteria growth with specific locations and salt concentrations.

2 THE CHURCH OF SAINT JEROME, GRANADA

The massive occurrence of salts in the interior of Saint Jerome Church is the most important and ubiquitous decay phenomenon, according to preliminary studies (Cardell & Rodríguez-Gordillo 1992).

The specific places where salts crystallize inside of the church is a consequence of the urban and geographic location of the building, and the precarious state of conservation of the construction materials in the exterior of the monument. The deterioration of such materials has been induced by original structural defects, inappropriate evacuation of rain water, occlusion of gargoyles by pigeon excrements and plants, etc. Salts crystallization occur in the zone of rising damp in the apse (east and west side) and the two adjacent rooms (Fig. 1), as well as in the apse, but up to a level of about 15 m high above the floor.

The nature, habits and aggregation forms of the salts depend mostly on the substrate where they crystallize (e.g. calcarenite, different types of mortars, brick and mural paintings) and also on the local internal conditions. The striking level of deterioration observed on the walls of Saint Jerome Church is due to the extremely sensitive response of paintings to salt weathering. These paintings cover all around the walls of the temple. Table 1 shows the major salts identified in the church.

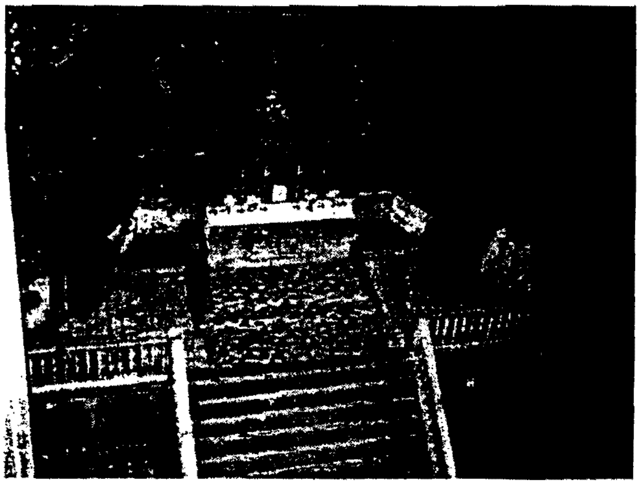

Figure 1. Retable of Saint Jerome Church.

Table 1. Major salts identified in Saint Jerome Church.

Carbonates	
Natron	$Na_2CO_3 \cdot 10H_2O$
Thermonatrite	$Na_2CO_3 \cdot H_2O$
Nacholite	$NaHCO_3$
Trona	$Na_3H(CO_3)_2 \cdot 2H_2O$
Sulfates	
Gypsum	$CaSO_4 \cdot 2H_2O$
Epsomite	$MgSO_4 \cdot 7H_2O$
Hexahydrite	$MgSO_4 \cdot 6H_2O$
Nitrates	
Nitratite	$NaNO_3$
Niter	KNO_3
Chlorides	
Halite	NaCl

2.1 *Low apse*

2.1.1 *East retable*

The retable shows fluffy sub-efflorescences and efflorescences composed of hexahydrite and epsomite. Dissolution–recrystallization cycles were observed (Fig. 2).

2.1.2 *West retable*

The main salts appearing in this area were magnesium and calcium sulfates: mostly hexahydrite and epsomite followed by gypsum as loose sub-efflorescences and efflorescences below and on gypsum mortar and lime–sand mortar (Fig. 3).

2.1.3 *East room*

Halite was the only salt present in efflorescences in spite of the nature of the substrate (rendering mortars of gypsum, gypsum and lime, and lime and sand). Halite crystallizes as a thick granular and opaque crust (2–3 cm) up to 3 m high on the walls. The great crystallization pressures originated the detachment and fall down of the mortars. On the floor, periodically cleaned with water, it was possible to observe the most common habits of fresh efflorescences that halite shows: starting from cubes (equilibrium form) towards prisms and acicular, hair like crystals, the so-called whiskers. They grow forming fluffy efflorescences as loose aggregates.

2.1.4 *West room*

Sulfates were the most abundant salts, namely epsomite, hexahydrite and gypsum, appearing as sub-efflorescences below rendering mortars of gypsum and lime, and lime and sand. These were coated by mural paintings composed of gypsum. Scarce halite was identified in this room. Due to the advanced deterioration of the paintings, in a recent past they were covered by a lime mortar, unfortunately. The aesthetic result is much worse, besides provoking the crystallization of nitrates (nitratite, niter) and carbonates (natron, trona and thermonatrite) efflorescences that reach up to 3 m high (Fig. 4).

Figure 2. East retable.

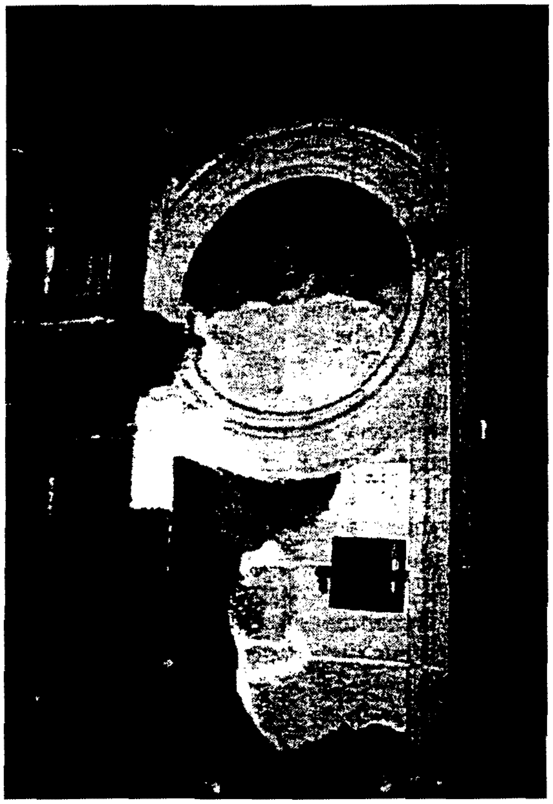

Figure 3. West retable.

Figure 4. West room.

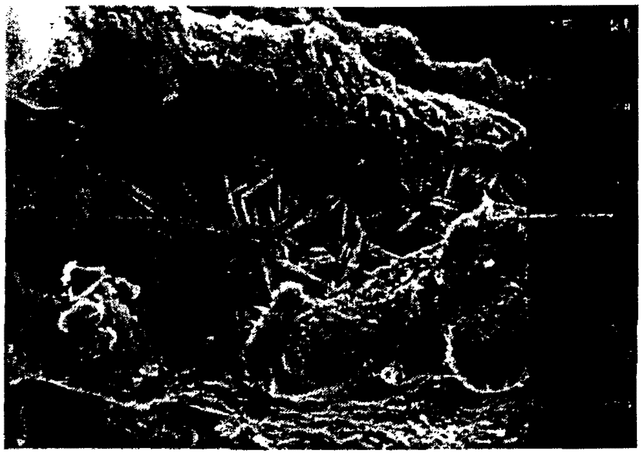

Figure 5. West room crystals.

Figure 5 shows a SEM micrograph with acicular crystals of niter and masses of nitratite and niter crystals.

At the floor level of the apse, salt crystallization is due to rising damp from ground water. The water supply must be continuous during enough time to produce the thick salt crust that, in the west room, arises about 3 m high. The origin of the damp is due to the location of the church; sloping down from south to north following an ancient stream, and with a little square full of trees in its north side.

2.2 *High apse*

The development of loose sub-efflorescences of crystals with granular habits exclusively composed of magnesium sulfate (epsomite and hexahydrite), and also gypsum, was massive. Epsomite was more abundant here than in the lower part of the temple. Less proportions of sodium carbonates (nacholite, trona) and sodium nitrate (nitrátite) were identified. The location of the salts was very punctual, crystallizing on the base of the stone polychromed sculptures placed in the triforium, and exclusively toward the ends, never in the center. All the decorative elements and walls were painted. The salt weathering effect is of such a magnitude that provokes the detachment, fall down of the paintings and the collapse of the calcarenite. The origin of the decay at this altitude is a consequence of rainwater infiltration, due to the defective water evacuation of the building. This has favored the percolation of water inside the church.

3 MATERIAL AND METHODS

3.1 *Location of samples*

Four samples representative of the different types of salts were studied in this paper. Location and composition is shown in Table 2.

3.2 *Enumeration and isolation of bacteria*

Efflorescences were collected in sterile tubes. To 1 ml of sterile distilled water 500 mg of each sample was added. The plates were inoculated with 100 µl of appropriated dilutions in the media Tryptic Soy Agar, Sigma, St. Louis, USA (TSA), starch-casein agar, starch-casein agar + 5% NaCl, malt-yeast extract agar, tap water agar and TSA with increasing concentrations of halite or epsomite (0.9, 3, 5, 10, 15 and 25%). The plates were incubated at 28°C for 48 h and counted.

Table 2. Samples, location and salts identified.

Sample	Location	Composition	Abundance
E-2	East retable	Epsomite	+++
		Calcite	++
		Hexahydrite	+
		Gypsum	+
W-5	West room	Nitratite	+++
		Epsomite	++
		Hexahydrite	+
		Niter	+
E-9	East room	Halite	++
W-10	West retable	Hexahydrite	+++
		Epsomite	+++
		Gypsum	++
		Calcite	++

3.3 *Characterization of the isolates*

After enumeration, individual colonies were isolated randomly and purified by streak plating onto TSA until pure culture plates were obtained. Isolates were characterized by morphological and physiological properties, the latter using standard microbiological methods, API and Biolog. Total cellular fatty acid methyl esters (FAME) were analyzed using the Microbial Identification System, Inc. Delaware, USA, (MIDI) in accordance with protocols for cultures grown on solid medium and instrument specifications recommended. This permitted automatic identification of bacteria by comparison with the MIDI Sherlock Standard Aerobe and Actin1 databases.

4 RESULTS

Numerous samples were taken from the different rooms and places, to investigate the bacteria associated to the efflorescences, however, in this paper only four representative samples are presented. They corresponded to the east part of the retable (E-2), composed of epsomite as the main salt followed by calcite and minor amounts of hexahydrite and gypsum; W-5, from the west room, composed of nitratite and comparatively minor amounts of epsomite and hexahydrite; E-9, from the east room composed of halite; and W-10 from the west side of the retable, composed of hexahydrite and epsomite as majority salts (Table 2).

Figure 6a shows the population of bacteria in the samples at increasing concentrations of sodium chloride, which is the salt used for isolation of halotolerant and halophilic bacteria. From all samples W-5 showed the high cfu. Interestingly the cfu in the medium with 0.9% sodium chloride was higher than without sodium chloride. In the samples E-2, E-9 and W-10 the cfu decreased as increased sodium chloride concentration and at 10% sodium chloride no growth was observed in E-2 and W-10. However, in sample E-9, composed of halite, cfu were evident up to 15% sodium chloride but in amounts considerably lower.

In Figure 6b sodium chloride was replaced by magnesium sulfate and the trend of the bacteria population was somewhat different. All samples showed growth at 15% magnesium sulfate, except sample E-9. In this sample, growth was very restricted at increasing magnesium sulfate concentrations, with no growth at 5%. This indicates that there is some specificity for magnesium or sodium in the respective populations. W-5 was also the sample with the high cfu counts, even at increasing magnesium sulfate concentrations, as those of 0.9% and 3% were higher than in the medium without salts.

The high counts obtained for sample W-5 could be related with the presence of nitrates together to magnesium salts. The data indicates that at salt concentrations between 3% and 5% appears to have a stimulatory effect, or that these concentrations are in the range of what the bacteria would consider optimum conditions for growth in deteriorated monuments. There is also a clear response

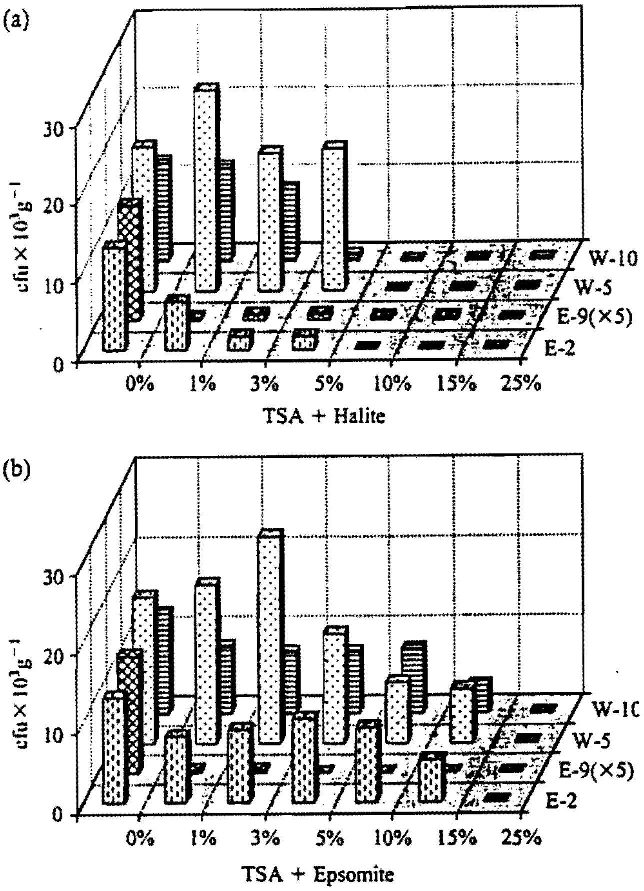

Figure 6. Enumeration of bacteria at increasing salt concentrations. Note that 0.9% concentration appears as 1%.

Table 3. Bacteria isolated from efflorescences.

Strain	Identification	Similarity Index (SI)
E-2.14	*Bacillus circulans*	0.521
E-2.15	*Bacillus* sp.	
E-2.17	NI*	
E-2.182	NI*	
W-5.3	NI*	
W-5.9	NI*	
W-5.10	NI*	
W-5.15	NI*	
W-5.26	*Bacillus pumilus*	0.602
W-5.31	*Bacillus circulans*	0.585
W-5.36	*Micrococcus luteus*	0.333
W-5.37	*Staphylococcus equorum*	0.466
W-5.201	NI*	
E-9.1	*Streptomyces* sp.	
E-9.15	*Bacillus licheniformis*	0.729
E-9.16	*Kocuria rosea*	0.363
W-10.3y	*Cellulomonas gelida*	0.390
W-10.3p	*Micrococcus luteus*	0.534
W-10.8	*Bacillus cereus*	0.721
W-10.11	NI*	
W-10.13	*Arthrobacter crystallopoietes*	0.571
W-10.15	NI*	
W-10.22	*Micrococcus luteus*	0.378
W-10.26	*Bacillus licheniformis*	0.680
W-10.29	*Bacillus megaterium*	0.717
W-10.72	NI*	

* NI: Not identified.

of the bacterial community to the presence of magnesium sulfate, which was adapted for growth. It was not so evident in the presence of sodium chloride, as indicated sample E-9 only composed of halite.

From these samples were isolated 26 bacteria, 8 *Bacillus* strains and 3 *Micrococcus* strains, which represented about 42% of the isolates (Table 3).

The abundance of *Bacillus* strains in efflorescences and salt deposits was already reported by Incerti et al. (1997) and Saiz-Jimenez & Laiz (2000). *Bacillus* can be considered a common genus in monuments deteriorated by salt effects.

5 DISCUSSION

Although the ecology and physiology of bacteria in underground mineral deposits (Cañaveras et al. 1999) and monuments deteriorated by effect of salt action is relatively unknown, the data from this and previous papers (Incerti et al. 1997, Saiz-Jimenez & Laiz 2000) indicate the continuous presence of halotolerant bacteria, particularly *Bacillus* species. This predominance is remarkable.

The abundance of bacilli could be explained by the osmotic adaptation of halophilic and halotolerant bacteria. In fact, bacteria are frequently exposed to stresses due to limitations and changes in nutrient availability, temperature, salinity, etc. and its persistence in the environment is to a great part determined by their availability to endure these stresses. While archaea tolerate high cytoplasmic concentrations of KCl, the KCl-type strategy, the organic-osmolyte (compatible-solute) type is widespread among aerobic eubacterial halophiles and represents a very flexible mode of adaptation making use of distinct stabilizing properties of compatible solutes (Galinski 1993). Most of the organic osmolytes responsible for osmotic balance (glycine betaine, ectoines, proline, N-acetylated diamino acids and N-derivatized glutamine amides) have been found in bacilli which would explain their abundance in efflorescences. Thus, the ability to synthesize ectoine is very common among aerobic heterotrophic eubacteria including Gram-positive cocci and *Bacillus* species; proline was originally considered the typical solute of halophilic *Bacillus* species, and a screening on halophilic/halotolerant bacilli and related species revealed that the majority of species produced ectoine, either alone or in combination with proline and/or N-acetylated diamino acids (Müller 1991).

Crystallization of salts in building materials produces a destructive effect. Under favourable conditions some salts may crystallize or recrystallize producing cracking, powdering and flaking. There are several hydrated phases of magnesium sulfate. The first one, precipitating from a saturated solution and at temperatures below 60°C, is epsomite. Dehydratation of epsomite originate hexahydrite. The predominance of one over other depends on the relative humidity. Halite, and other hygroscopic salts, can retain considerable quantities of moisture when entrapped in the building material, leaving the areas of salt concentrations moist throughout the entire year and thus favoring microbial growth. In turn, these microorganisms can mediate the crystallization process.

It is well-known that bacteria are able to precipitate calcite and other minerals such as aragonite, struvite, bobierrite, apatite, etc. (Rivadeneyra et al. 1992). In this respect, the production of calcite crystals by soil bacteria has been considered a general phenomenon. Many bacilli including *B. pumilus*, *B. subtilis*, *B. megaterium*, *B. cereus*, *B. bulgaricus*, and *B. globigii* form calcite crystals on solid media containing sodium acetate (Boquet et al. 1973).

The formation of calcium carbonate by some moderately halophilic bacteria was also observed by Del Moral et al. (1987). Optimum salt concentration for precipitation was 10%, and it is noteworthy that in most cases the higher counts were found around this concentration. Rivadeneyra et al. (1993) found that 26 moderately halophilic strains of *Bacillus* isolated from a saline soil precipitated magnesium calcite, aragonite, monohydrocalcite and dolomite in varying proportions. Recently Cañaveras et al. (1999) studied the formation of minerals in caves. The presence of some minerals was highly suggestive of a microbial-mediated precipitation and it was indicated that microbial activity may play a direct or indirect role in the precipitation of hydromagnesite deposits in the Cave of Altamira.

A conclusion derived from this and other studies (Saiz-Jimenez & Laiz 2000) is that in monuments deteriorated by the effect of salts, enumeration of bacteria yielded higher counts at increasing concentrations of salts than when enumeration was carried out in media without salts. Probably, culture media with moderate to high salt concentrations reproduced better the original niche than media based exclusively on organic substrates, and, therefore, a more realistic picture of the microbial communities can be obtained.

Magnesium sulfate-containing media gave higher counts than sodium chloride-containing media, particularly in environments where efflorescences or mineral deposits were based on magnesium salts. De Medicis (1986) stated that magnesium may play a role in haloadaptation. The data indicate that enumeration based exclusively on media rich in organic substrates are biased and that knowledge on the nature of the efflorescences and/or salts in deteriorated monuments is required for preparing the appropriate media for enumeration. Therefore, according to Saiz-Jimenez & Laiz (2000) it is proposed that, in the studies on microbial communities in deteriorated monuments, should be applied the protocols involving the use of media with high concentrations of sodium chloride (up to 25%) and 2% magnesium sulfate for environments in which the predominant cation is sodium, and media with high concentrations of epsomite for environments in which the predominant cation is magnesium.

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REFERENCES

Boquet, E., Boronat, A. & Ramos-Cormenzana, A. 1973. Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. *Nature* 246: 527–528.

Cardell, C. & Rodriguez-Gordillo, J. 1992. Formacion de sales en materiales petreos (Monasterio de San Jeronimo, Granada). Origen y procesos de deterioro asociados. In *III Cong. Geol. España y VIII Cong. Latinoamericano Geol. 3*: 46–48.

Cardell, C. 1998. *Cristalización de sales en calcarenitas: Aplicación al Monasterio de San Jerónimo.* Ph.D. Thesis. University of Granada, Granada.

Cañaveras, J.C., Hoyos, M., Sanchez-Moral, S., Sanz-Rubio, E., Bedoya, J., Soler, V., Groth, I., Schumann, P., Laiz, L., Gonzalez, I. & Saiz-Jimenez, C. 1999. Microbial communities associated with hydromagnesite and needle-fiber aragonite deposits in a karstic cave (Altamira, Northern Spain). *Geomicrobiology Journal* 16: 9–25.

De Medicis, E. 1986. Magnesium, manganese and mutual depletion systems in halophilic bacteria. *FEMS Microbiological Reviews* 37: 137–143.

Del Moral, A., Roldan, E., Navarro, J., Monteoliva-Sanchez, M. & Ramos-Cormenzana, A. 1987. Formation of calcium carbonate crystals by moderately halophilic bacteria. *Geomicrobiology Journal* 5: 79–87.

Galinski, E.A. 1993. Compatible solutes of halophilic eubacteria: molecular principles, water-solute interaction, stress protection. *Experientia* 49: 487–496.

Hof, T. 1935. As quoted in Ventosa, A., Nieto, J.J. & Oren, A. 1998. Biology of moderately halophilic aerobic bacteria. *Microbiology and Molecular Biology Reviews* 62: 504–544.

Incerti, C., Blanco-Varela, M.T., Puertas, F. & Saiz-Jimenez, C. 1997. Halotolerant and halophilic bacteria associated to efflorescences in Jerez Cathedral. In F. Zezza (ed.), *Origin, mechanisms and effects of salts on degradation of monuments in marine and continental environments*: 225–232. Protection and Conservation of the European Cultural Heritage Research Report no. 4.

Müller, E. 1991. *Kompatible Solute und Prolingewinnung bei halophilen und halotoleranten Bacilli.* Ph.D. Dissertation. Universität Bonn, Bonn.

Norton, C.F. 1992. Rediscovering the ecology of halobacteria. *ASM News* 58: 363–367.

Oren, A. 1994. The ecology of the extremely halophilic archaea. *FEMS Microbiological Reviews* 13: 415–440.

Rivadeneyra, M.A., Perez-Garcia, I. & Ramos-Cormenzana, A. 1992. Struvite precipitation by soil and fresh water bacteria. *Current Microbiology* 24: 343–347.

Rivadeneyra, M.A., Delgado, R., Delgado, G., del Moral, A., Ferrer, M.R. & Ramos-Cormenzana, A. 1993. Precipitation of carbonates by *Bacillus* sp. isolated from saline soils. *Geomicrobiology Journal* 11: 175–184.

Rodriguez-Valera, F. 1988. *Halophilic bacteria*. Boca Raton: CRC Press.

Rölleke, S., Muyzer, G., Wawer, C., Wanner, G. & Lubitz, W. 1996. Identification of bacteria in the biodegraded wall painting by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology* 62: 2059–2065.

Saiz-Jimenez, C. & Laiz, L. 2000. Occurrence of halotolerant/halophilic bacterial communities in deteriorated monuments. *International Biodeterioration and Biodegradation* 46: 319–326.

Vreeland R.H. & Hochstein L.I. 1993. *The biology of halophilic bacteria*. Boca Raton: CRC Press.